THE GROWTH OF VACCINIA VIRUS IN THE CHORIO-ALLANTOIS OF THE DEVELOPING CHICK EMBRYO AND THE PRODUCTION OF COMPLEMENT-FIXING ANTIGEN AND HAEMAGGLUTININ

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(With 7 Figures in the Text)

This study of the growth curve of vaccinia virus in the chorio-allantois of the chick embryo was undertaken to obtain data which might help to elucidate the mechanism of multiplication of the virus, and to determine whether a growth cycle was demonstrable. The development of complement-fixing antigen and haemagglutinin in relation to virus growth has also been investigated.

MATERIALS AND METHODS

Virus. Vaccine lymph prepared at the Lister Institute was passed once through testis, and thereafter intradermally, in rabbits. A second sample of the virus, received as an elementary body suspension from rabbit skin, was also passed intradermally in rabbits. From each skin passage an elementary body suspension was made, by the method of Macfarlane & Salaman (1938) or that of Hoagland, Smadel & Rivers (1940).

A strain of cowpox was isolated from a human case on the chorio-allantois and maintained by further passages on this tissue.

All virus suspensions were stored at -20 to $-38°$ C. in small amounts in order to avoid repeated freezing and thawing.

Antiserum. Antivaccinia rabbit sera were prepared by intradermal and later intravenous inoculation of rabbit elementary body suspensions. The sera which contained neutralizing, complement-fixing and haemagglutinin-inhibiting antibodies were stored at $-20°$ C.

Titration of virus. Virus was diluted in M/250 McIlvaine's buffer, pH 7·4, which was shown to give the same titration end-points as dilution in Hartley's broth or Tyrode's solution. Eggs were usually incubated for 12–13 days but sometimes for 11 or 14 days, and four or more were inoculated with 0·05 ml. of each ten-fold dilution of virus directly on the chorio-allantoic membrane. They were then incubated at 36·5° C. for 48 hr. when pock counts were done.

Recovery of virus from infected chorio-allantoic membranes

Infected membranes were disintegrated in a grinding tube (Griffith's tube) made from a test-tube of 25 mm. diameter. The head of a pestle was blown from glass tubing and ground to fit the base of the test-tube. The opposing surfaces were

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roughened, and the sideways fit of the pestle in the tube was loose so as to avoid pieces of tissue collecting above the head. Up to three chorio-allantoic membranes could be ground together satisfactorily; grinding was by hand for 15 min. No intact cells, or at most very few, were detectable microscopically in the ground material, which consisted of granular debris. Further grinding did not increase the yield of virus. The amount of virus obtained by this procedure was greater than by grinding frozen membranes with a pestle and mortar, by grinding them in a dry mortar without abrasive at room temperature or by grinding them in a dry mortar with fine glass beads which were themselves broken into very fine particles. Disintegration of membranes in an Ato-Mix blender running at full speed for 2 min. yielded somewhat more virus than grinding in a Griffith's tube. The metal head of the machine was cooled at $-20°$ C. before putting into it 25 ml. of chilled Tyrode's solution, which was the minimum volume needed by the machine, and five chorio-allantoic membranes. After running for 2 min. the temperature was about 21° C. No intact cells were detectable microscopically. This method was not used as a routine in view of the volume of diluent required.*

GROWTH-CURVE EXPERIMENTS

Eggs after incubation for 12–13 days were inoculated directly on the dropped chorio-allantoic membrane, incubated further at 36·5° C. and harvested at intervals for titration. Those harvested 'immediately' were separately inoculated, sealed and opened. The shell was cut round with scissors just below the inoculated area of membrane, and the portion of shell thus separated was removed and inverted. The membrane was detached with forceps from the marginal area of this piece of shell and allowed to drain into it. The membrane and liquid were collected and titrated separately. A pool from three or four eggs was used for each titration. The pools were stored at $-20°$ C. At the end of an experiment all the pools of membranes were thawed, each was ground and made up to 1 ml. per membrane with M/250 McIlvaine's buffer, pH 7·4; 2 ml. of a 1/10 dilution of each suspension was made for subsequent titration and divided into three portions which were stored at $-20°$ C.; the liquid and some of the inoculum had been stored similarly in three portions. A preliminary titration of one portion of all these materials was made with a few eggs as a guide; the second portion was then titrated at the appropriate dilution and the third portion was available for further titration if required. This ensured that all the materials from one experiment were titrated on the same batch of eggs.

The remainder of the undiluted suspension was centrifuged to deposit the virus, and the non-infective supernatant fluid was stored at $-20°$ C. to be tested for complement-fixing antigen and haemagglutinin.

Complement fixation. Two M.H.D. of complement (titrated without antigen), 5 M.H.D. haemolysin and 2·5% of packed sheep cells were used. Fixation was overnight at 4° C.; after adding the haemolytic system the tubes were kept in

* Since this work was done, Measuring and Scientific Equipment Ltd. have produced smaller containers for the Ato-Mix machine which are advantageous for some purposes.

a water-bath at 37° C. for 30 min. then placed at 4° C. and read when the cells had settled. A reference antigen was prepared by the method of Casals (1949) from chorio-allantoic membranes with confluent lesions disintegrated in an Ato-Mix machine. It was standardized against three minimal reacting doses of a selected serum which had been titrated against a crude ground suspension of heavily infected chorio-allantoic membrane shown to be non-anticomplementary. The reference antigen also was completely non-anticomplementary and non-infective and had a titre of 1/64–1/128. It was stored at −20° C. without preservative and showed little change in titre for 8–10 weeks, but after that time required replacing as the titre was apt to decrease suddenly. Sera used in tests for assessing the amount of antigen in membranes from the growth-curve experiments were standardized against four minimal reacting doses of reference antigen. There was no fixation when they were tested against material from normal chorio-allantoic membranes.

In assessing antigen in material, prepared as already described, from membranes harvested in the growth-curve experiments, two sets of doubling dilutions were made. To one set three minimal reacting doses of serum and 2 M.H.D. complement were added; the other set received 1½ M.H.D. complement only and was a control for anticomplementary activity of the antigenic material under test. A serum control, with complement only, was always set up. And to ensure continuity of values the reference antigen was titrated on each occasion so that any deviation in sensitivity of the test would be detected.

Haemagglutinin. To doubling dilutions of the test material were added an equal volume of 1/100 normal rabbit serum (to inhibit non-specific haemagglutination; Stone & Burnet, 1946) and 1 % washed fowl red blood cells known to be readily agglutinable. The tubes were placed in an incubator at 37° C. for 1 hr. and shaken after 30 min. The end-point was the last tube showing a central button of cells with a small but definite scattering of agglutinated cells around it. A potent reference haemagglutinin was prepared by grinding chorio-allantoic membranes which had confluent lesions, adding 0·5 ml. saline per membrane, clearing the suspension of virus in a centrifuge and storing the supernatant fluid at −20° C. This was always included in an experiment and ensured by its constant titre a quantitative continuity in results.

RESULTS

Adsorption of virus by the chorio-allantoic membrane

In following quantitatively the fate of virus inoculated on the chorio-allantoic membrane it is necessary to take into account initially two factors. The first factor is a change in the infectivity of the virus which occurs almost immediately after its contact with the membrane and results in a marked increase in titre of the recoverable virus. This will be referred to as enhancement of infectivity or merely 'enhancement' and will be considered more fully in another paper. The second factor is the rate of adsorption or fixation of the virus by the membrane and its distribution between the membrane and the tissue fluids.

In the three experiments illustrated in Fig. 1 the dose of virus inoculated on the chorio-allantois was calculated in terms of pocks from a titration of the inoculum in eggs. It will be seen that in each experiment the total amount of virus recovered immediately after inoculation was in excess of that inoculated; the increase was from 5- to 22-fold (in other experiments often 2- to 4-fold) and most of the virus was in the liquid. The amount of virus in the membrane alone was about equal

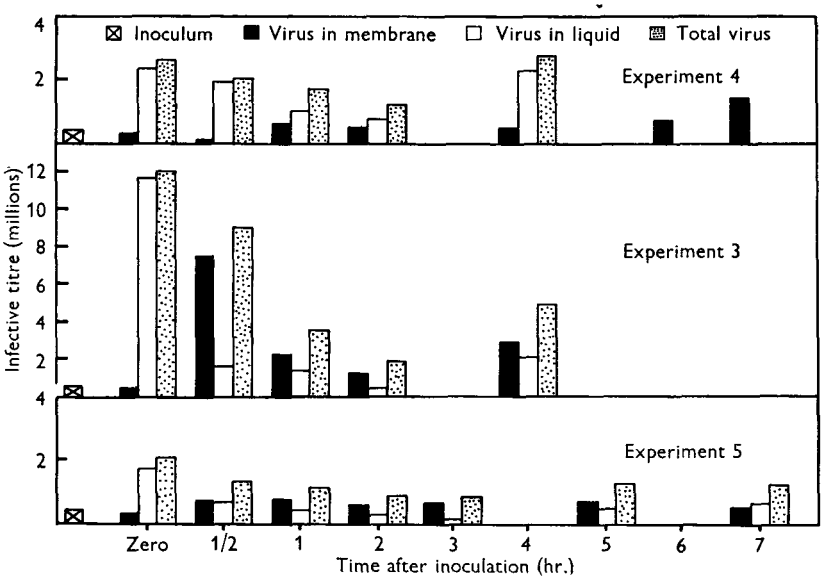

Fig. 1. Behaviour of virus inoculated on the chorio-allantoic membrane during the first 7 hr.

to the inoculum. In subsequent titrations it is seen that the distribution of virus between membrane and liquid changed, the virus becoming adsorbed on the membrane and disappearing from the liquid. The peak amount of virus in the membrane was noted at $\frac{1}{2}$ hr. (Exp. 3) and at 1 hr. (Exps. 4 and 5), but these times are approximate owing to the intervals between titrations.

Reduction of infectivity

The point of peak concentration of virus in the membrane was followed by a gradual reduction in titre before multiplication began (Fig. 1). The lowest point appeared to be at about 2 hr. after inoculation in two experiments and about 4 hr. in another. The amount of virus at this point was 20–80 % of the peak and 2–4 times the amount inoculated. There was never anything approaching extinction of infective virus.

It will be noted also in Fig. 1 that the total amount of virus began to decrease soon after inoculation and continued to do so steadily throughout the period when virus was accumulating on the membrane.

Other experiments have yielded similar results. Enhancement occurred in nine of fifteen experiments when membranes only were titrated 30 min. after inoculation and in all of three others when both membranes and liquid were titrated.

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In twelve experiments the highest level of virus in the membrane was noted between ½ and 2 hr. after inoculation (usually about 1 hr.) and the lowest level between 2 and 4 hr.

The beginning of multiplication of virus

The time at which virus began to multiply can be inferred from the point at which the curve of total virus titres began to rise. Some virus multiplication may have begun before this point during the later part of the downward trend but was obscured by the continuing loss; similarly, during the early part of the rise some loss of virus may have continued. It is evident, however (Fig. 1), that in Exps. 3 and 4 there was some increase of virus between 2 and 4 hr. and in Exp. 5 between 3 and 5 hr. after inoculation.

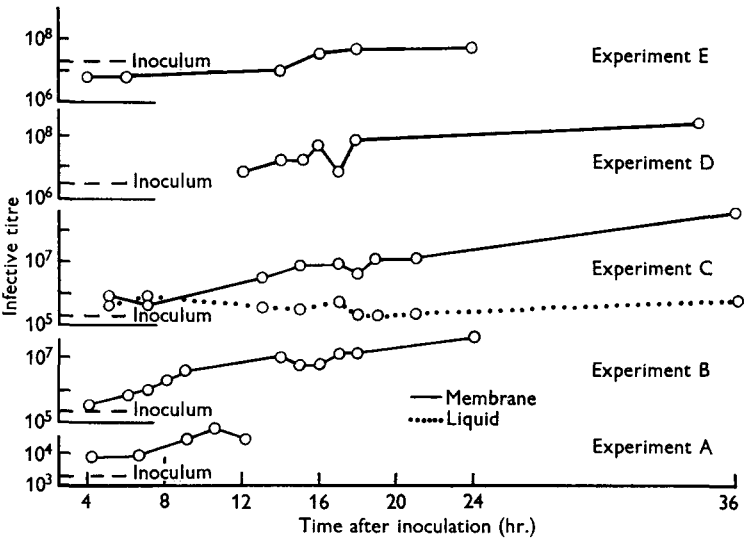

Fig. 2. Increase of virus in the chorio-allantoic membrane from 4 to 36 hr. after inoculation.

Growth of virus in the chorio-allantois

In experiments covering the period 4–36 hr. after inoculation a continual and gradual increase in virus occurred in the membrane (Fig. 2). In some experiments there was some unevenness at 16–18 hr., the significance of which is uncertain, but there was no evidence supporting a stepwise increase in titre. During this period virus in the liquid did not increase in the one experiment where this was tested.

The rate and amount of multiplication of virus in the chorio-allantois was markedly affected by the size of the inoculum (Figs. 3–5). It will be seen that after three widely differing inocula the same final concentration of virus in the membrane, approximately $10^{7\cdot5}$, was reached in about 36 hr. and maintained to the 4th day. This represented an increase of 6·9-, 125- and 69,000-fold for inocula containing $10^{6\cdot7}$, $10^{4\cdot8}$ and $10^{2\cdot2}$ infective units respectively. These findings are representative of eight similar experiments.

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The growth of cowpox virus in the chorio-allantois

The multiplication of cowpox virus, for comparison with vaccinia, was followed in one experiment as shown in Table 1. Enhancement of virus occurred immediately after its contact with the membrane. The rate of multiplication appeared to be similar to vaccinia, likewise the maximum concentration $10^{7\cdot8}$. The virus content of the liquid did not rise during the most active phase of multiplication in the membrane, again resembling vaccinia, but it did rise to some extent after 3 days when virus seemed to be passing from the membrane to the liquid.

Table 1. *Development of cowpox virus in the chorio-allantois following an inoculum of* $10^{3\cdot5}$ *infective particles*

Time after inoculation (days)	Infective particles per membrane	Infective particles in liquid
Immediate	$10^{3\cdot8}$	$10^{4\cdot2}$
1	$10^{6\cdot6}$	$10^{4\cdot2}$
$2\frac{3}{4}$	$10^{7\cdot8}$	$10^{4\cdot9}$
3	$10^{7\cdot8}$	$10^{4\cdot5}$
$3\frac{3}{4}$	$10^{6\cdot7}$	$10^{5\cdot3}$

The development of complement-fixing antigen in relation to the multiplication of virus

The possibility that a non-infective stage of virus in a growth cycle, if it occurred, might be antigenic and separable from elementary bodies has been investigated. The antigenic material was prepared from the suspensions of ground infected membranes which were titrated in the growth-curve experiments, as described in the section on methods.

Complement-fixing antigen could not be detected immediately after inoculation at the time of enhancement or before onset of multiplication during the period when the titre of virus was falling. Its development was associated with multiplication of virus; the threshold of its detection corresponded with an infective titre of $10^{5\cdot5}$ to $10^{6\cdot0}$. With a large inoculum, $10^{6\cdot3}$ (Fig. 3), antigen could be detected earlier (in 6 hr.—not in 30 min.) than after a relatively small inoculum, $10^{2\cdot2}$ (Fig. 5), when there was no detectable antigen for more than 12 hr.; an inoculum of intermediate size (Fig. 4) gave an intermediate result. The curves of increase of antigen suggested that the bulk of virus undergoing multiplication was the determining factor in the appearance and increase of antigen in the membrane, rather than the duration of growth of virus or the number of times the inoculum had reproduced itself. Probably some antigen was elaborated from the beginning of virus growth, but at first was too small in amount to be detectable. It may have accumulated as virus continued to grow. There was nothing in these results to indicate that antigen was produced in a stepwise manner with sudden increases in titre.

The results shown in Figs. 3–5 are representative of eight similar experiments. In one experiment in which the liquid was examined for antigen, relatively little was found at any time up to 21 hr. This liquid had a low infectivity.

An analysis has been made of results when the same material was titrated both for infectivity and for antigen; there were 125 such results from sixteen experiments.

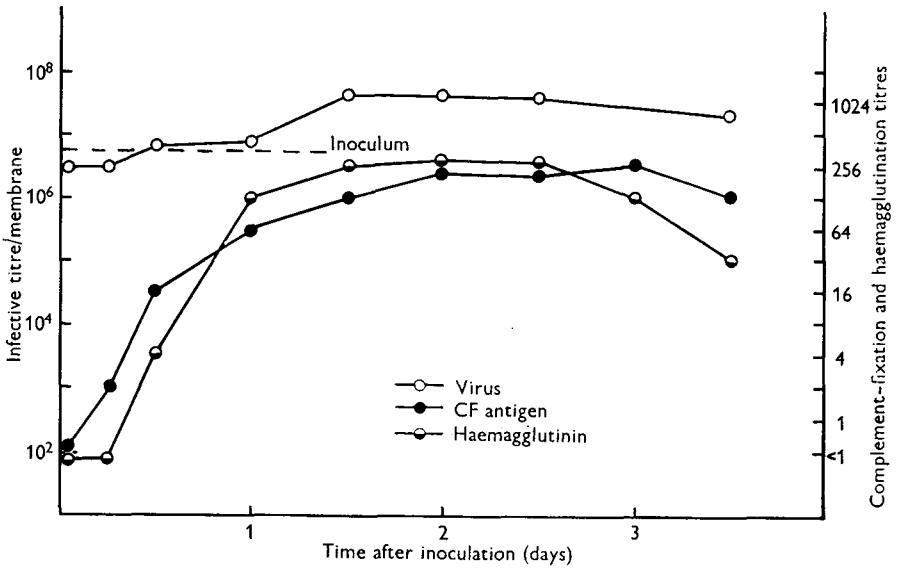

Fig. 3. Increase of infectivity, complement-fixing antigen and haemagglutinin in the chorio-allantoic membrane after a large inoculum.

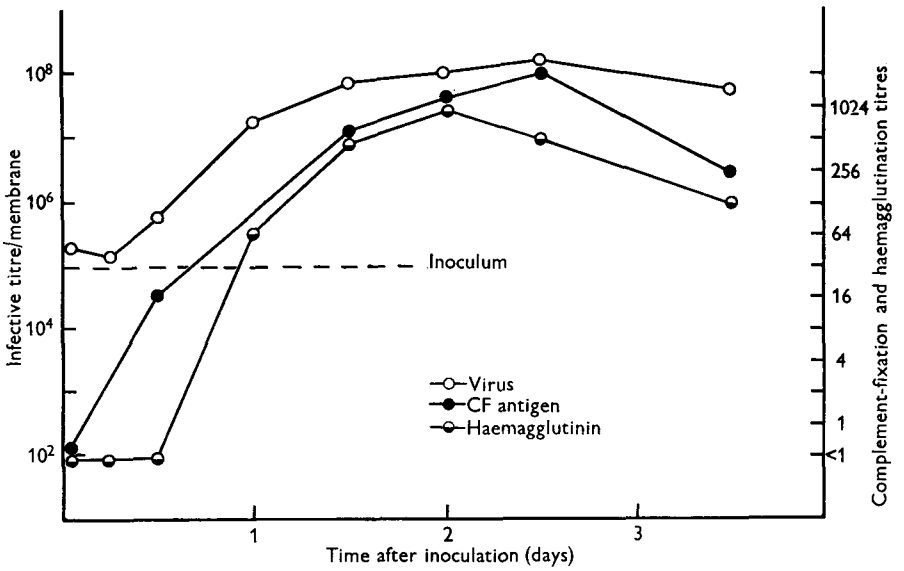

Fig. 4. Increase of infectivity, complement-fixing antigen and haemagglutinin in the chorio-allantoic membrane after an inoculum of medium size.

In Fig. 6 the antigen titre has been plotted against the geometric mean of infectivity (pock counts) of the corresponding samples. The points fall on a straight line, thus supporting a direct relationship between number of elementary bodies

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(infective units) and amount of antigen. The graph also shows that antigen was not detectable by the technique employed unless the infective titre was at least $10^{5.5}$ to $10^{6.0}$.

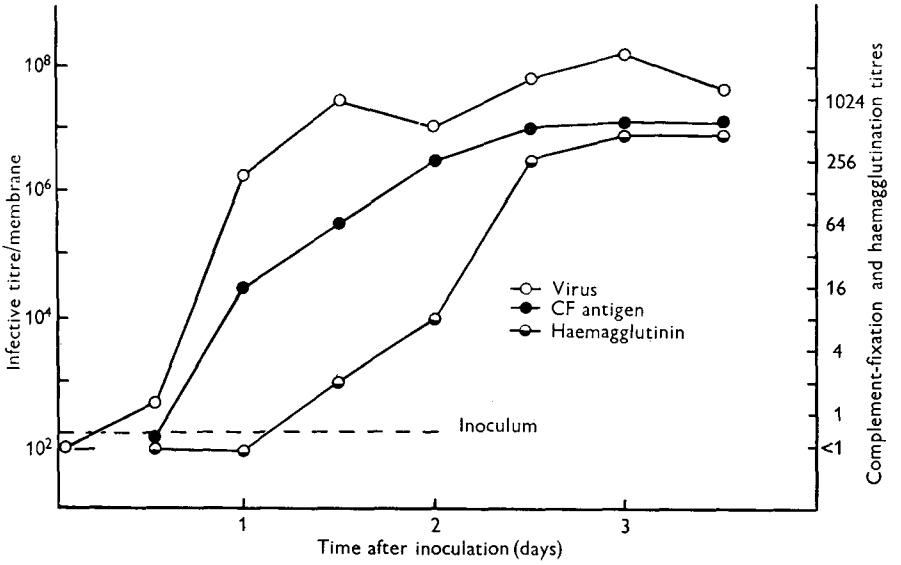

Fig. 5. Increase of infectivity, complement-fixing antigen and haemagglutinin in the chorio-allantoic membrane after a relatively small inoculum.

The development of haemagglutinin in the infected chorio-allantoic membrane

The findings which have been stated for complement-fixing antigen apply almost without alteration to haemagglutinin (Figs. 3–5). The only difference was that the level of detection of haemagglutinin was related to a slightly larger amount of virus, the minimal infective titre being $10^{6.5}$–$10^{7.0}$, so that haemagglutinin appeared somewhat later in the growth curve. Numerically the findings are similar to those of Briody & Stannard (1951), who gave the figure as 10^6.

In Fig. 7 the titre of haemagglutinin in fifty-five samples from thirteen experiments has been plotted against the geometric mean of the infective titres of the corresponding samples. The results are similar to those for complement-fixing antigen (Fig. 6) in showing a direct relationship between amount of haemagglutinin and number of infective particles.

DISCUSSION

Experiments of the type described here are not ideal for making precise deductions regarding the nature or rate of multiplication of vaccinia virus in individual cells and the release of virus from them. The overall picture of changes in the amount of virus and some virus products in relation to time, which can be obtained by examining the chorio-allantoic membrane, is a composite one representing a number of separate processes which proceed concurrently but which are not necessarily synchronized in different cells.

The analysis presented in this paper of what happens when vaccinia virus is inoculated on the chorio-allantoic membrane indicates that the situation is more complex than has been noted heretofore. The two new factors which have to be taken into account are enhancement of infectivity and the distribution of virus between the liquid which drains from an infected membrane and the membrane itself.

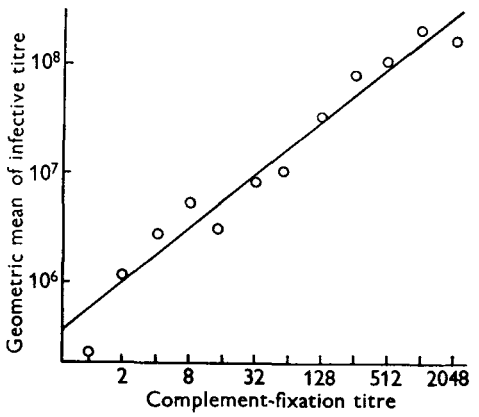

Fig. 6. Relation of infective and complement-fixing titres of material from chorio-allantoic membranes.

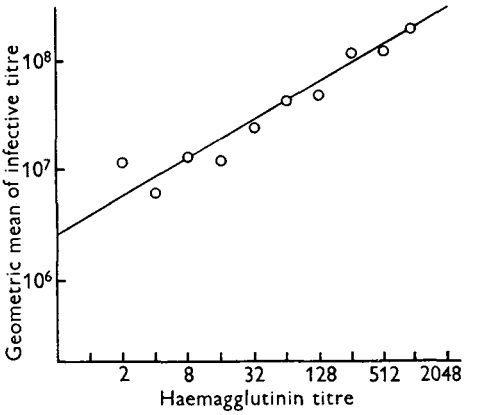

Fig. 7. Relation of infective and haem-agglutinin titres of material from chorio-allantoic membranes.

What occurs may be stated in general terms. Virus when it comes in contact with chorio-allantoic membrane becomes enhanced in infectivity almost at once, so that its infective titre is increased considerably. A large part of the virus immediately after inoculation is found in the liquid. The titre of the liquid begins to fall almost at once and continues to do so for 2–3 hr., apparently because virus in it is becoming attached to the membrane. Some virus is found in the membrane immediately after inoculation, and the amount continues to rise as virus passes to it from the liquid, presumably becoming adsorbed to cells of the membrane.

However, soon after inoculation, the total amount of virus begins to decrease, and this can be accounted for by disappearance of virus which has become attached to the membrane. *In vitro* studies (Maitland & Magrath, to be published) have shown a similar disappearance of virus from the membrane. Although some virus may spread to other parts of the egg and escape detection, the amount thus lost was found to be small in relation to the amount in the membrane. The actual titre of virus in the membrane during approximately the first 2–3 hr. after inoculation will thus depend on a balance between amount of enhancement, rate of adsorption of virus from liquid to membrane and rate of disappearance from the membrane; the peak occurred usually about an hour after inoculation, but this has no special significance.

At first the amount of virus being adsorbed by the membrane exceeds the amount which is disappearing, but when disappearance exceeds adsorption the

titre of virus in the membrane begins to fall. In 2–3 hr. the virus reaches its lowest level in the membrane and, as this is usually the case also with the liquid, it is the point of lowest total amount of virus. The curve of total virus then begins to rise. This increase has been detected between 2 and 4 hr. and between 3 and 5 hr. The end of the phase of disappearance may overlap and mask the beginning of multiplication. Interpretation is made more difficult by the probability that the events in all cells do not coincide in time. All the cells may not be infected simultaneously, and the metabolic activities of infected cells may be unequal. However, there is evidence that some virus had multiplied 4 or 5 hr. after inoculation. And it is clear that in order to detect the earliest increase, the total virus, that is, in both membrane and liquid, should be measured. On further incubation virus increases gradually in the membrane and appears to keep at about the same level in the liquid.

The experiments do not afford any information on the release of virus from individual cells. The 'enhancement' effect may operate on new virus, but this is not known. Virus released from infected cells and adsorbed on non-infected cells may disappear in part, similarly to the enhanced inoculated virus.

The time relationships noted in these experiments may possibly differ somewhat in other experiments, particularly those *in vitro*, and might be affected by differences in the virus used as inoculum, the state of the tissue and the conditions of incubation.

As with other systems used for virus multiplication there was an upper limit of virus concentration, and when it was reached multiplication ceased. What determines this is not known. When small inocula were used the increase was very much greater than that after large inocula, but in each case the maximum was attained in about the same time.

The meaning of enhancement is not at present certain. It may indicate a change in individual elementary bodies affecting such fundamental properties as infection, adaptation and mechanism of growth. On the other hand, it may mean no more than dispersion of clumps of elementary bodies, with a consequent increase in number of infective units. This matter is being investigated.

The explanation of the early loss of virus is not clear. The loss was not so great as to suggest that it necessarily indicated a non-infective stage in a growth cycle. At the point of lowest level, considering total virus, there was still 20–80 % as much of virus as was present in membrane and liquid immediately after inoculation (i.e. the amount which indicated enhancement) and 2–4 times the amount of the inoculum, as determined by titrating it directly on the chorio-allantoic membrane.

It is not possible to draw any firm conclusions regarding the relation between partial disappearance of virus and mechanism of multiplication. The virus which grew may be the virus which did not disappear. On the other hand, there is nothing to contraindicate that it was the virus that disappeared which eventually multiplied, though to sustain this view positive evidence would be required.

The results reported here do not agree with those of Briody & Stannard (1951), who noted a more rapid and marked disappearance of virus in the chorio-allantois and a stepwise increase at about 8 and 16 hr. after inoculation. They used egg-

passaged virus as inoculum, titrated virus in the membrane only and did not analyse the behaviour of virus in the detail reported in this paper.

The growth curve reported by Anderson (1954) for virus in the chorio-allantoic membrane dealt with virus in the membrane only and the possibility of enhancement was not considered. The results are therefore difficult to relate to those reported here. The lowest amount of virus in the membrane was reached in 9 hr. and thereafter virus increased gradually. No stepwise increase was noted. From this and other experiments the conclusion was drawn that there was an 'eclipse phase' of virus which lasted 9 hr. The implication was that virus went through a non-infective stage before multiplication, but, while this is one possible interpretation of the experiments, further work is required before such a conclusion can be firmly established.

Forsyth, Cook & Irons (1954) in a preliminary communication noted a rapid decrease of infectivity following inoculation of the chorio-allantoic membrane and a non-infective period lasting 4–14 hr., during which no virus could be recovered from the membrane. It appears that virus in the liquid was not titrated and that in some respects their results differed from those reported here.

Lépine, Wielgosz & Reinie (1951) studied growth curves from 12 hr. onwards after infecting the chorio-allantoic membrane and titrated virus by intradermal inoculation of rabbits. Their experiments were not concerned with the points raised in this paper.

The data relating to the production of 'soluble' complement-fixing antigen and haemagglutinin are markedly similar in that each is related directly to the number of infective units of virus. The exact way in which haemagglutinin or soluble complement-fixing antigen is produced is not understood, but each appears during the growth of virus in the cell; they may not be produced in an identical manner. The main difference noted between them was the larger number of infective units of virus associated with the threshold of detection of haemagglutinin and consequently its appearance somewhat later in the growth curve, but this difference did not necessarily indicate that they are separate entities as the sensitivity of the tests used for detecting them may differ. Haemagglutinin may possibly have been acting as a complement-fixing antigen, but even if this were so other complement-fixing antigens may have been present. The matter requires further investigation.

There was no evidence that haemagglutinin or complement-fixing 'soluble' antigen represented a non-infective phase of virus in a developmental cycle or that they increased in a stepwise manner. The mechanism of their production is uncertain, but the results suggested that they accumulated as virus continued to multiply rather than that they were used up to elaborate new elementary bodies and reformed in larger quantities with each increment of virus. The experiments do not, however, provide a clear indication of what happens in single infected cells, and therefore do not rule out entirely the idea of a non-infective phase or stepwise production of virus, but they do throw considerable doubt on conclusions in favour of this view which have been noted by others, based on infection of chorio-allantoic membranes.

SUMMARY AND CONCLUSIONS

1. Elementary body suspensions of vaccinia virus placed on the chorio-allantoic membrane of the developing chick embryo showed immediately an enhanced infectivity which may amount to a several-fold increase in titre.

2. In studying the growth curve of vaccinia virus in the chorio-allantois the distribution of virus between the liquid and the membrane had to be taken into account.

3. Some virus disappeared after inoculation, but this did not necessarily indicate a non-infective phase in a growth cycle.

4. Virus began to increase in 4–5 hr. and proceeded without stepwise increments, reaching a maximum in about 36 hr.

5. Haemagglutinin and complement-fixing 'soluble' antigen were formed during the growth of virus, and the titre of each was directly related to the amount of virus. They were detectable when the titre of virus reached respectively $10^{6\cdot5}$–$10^{7\cdot0}$ and $10^{5\cdot5}$–$10^{6\cdot0}$ pocks per ml.

6. Haemagglutinin may have been acting as a complement-fixing antigen.

7. Neither haemagglutination nor complement-fixation provided evidence for the occurrence of a non-infective phase of virus as part of a growth cycle.

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